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RNA/Protein Purification 96-Well Kit Product Insert

Product # 37900

Norgen's RNA/Protein Purification 96-Well Kit provides a rapid method for the high throughput isolation and purification of total RNA and proteins simultaneously from a single sample of cultured animal cells, small tissue samples, blood, bacteria, yeast, fungi or plants. It is often necessary to isolate total RNA and proteins from a single sample, such as for studies of gene expression including gene silencing experiments, mRNA knockdowns or experiments correlating RNA and protein expression levels. Traditionally the RNA and proteins would be isolated from different aliquots of the same sample, however this novel technology will allow for their simultaneous isolation from the same sample. This will not only save time, but will also be of a great benefit when isolating RNA and proteins from precious, difficult to obtain or very small samples. Furthermore, gene expression analysis will be more reliable since the RNA and proteins are derived from the same sample, therefore eliminating inconsistent results.

Norgen's Purification Technology

RNA Purification

Purification is based on 96-well column chromatography using Norgen's proprietary resin as the separation matrix. The purification could be performed on either a vacuum manifold or using centrifugation. The process involves first lysing the cells or tissue of interest with the provided Lysis Solution (please see the flow chart on page 4). The Lysis Solution contains detergents, as well as large amounts of a chaotropic denaturant that will rapidly inactivate RNases and proteases that are present. Alcohol is then added to the lysate, and the solution is loaded onto a 96-Well Filter Plate. Norgen's resin binds nucleic acids in a manner that depends on ionic concentrations, thus only the RNA will bind to the column while the proteins are removed in the flowthrough. The bound RNA is then washed with the Wash Solution in order to remove any impurities, and the purified RNA is eluted with the Elution Solution.

The kit purifies all sizes of RNA, from large mRNA and ribosomal RNA down to microRNA (miRNA) and small interfering RNA (siRNA). The purified RNA is of the highest integrity, and can be used in a number of downstream applications including real time PCR, reverse transcription PCR, Northern blotting, RNase protection and primer extension, and expression array assays.

Protein Purification

The proteins that are present from the initial flowthrough can now be loaded directly onto an SDS-PAGE gel for visual analysis. Alternatively, the protein samples can be further purified using the 96-Well Filter Plate provided with the kit. After the RNA has been eluted from the 96-Well Filter Plate, the flowthrough is then pH adjusted and loaded back onto the plate in order to bind the proteins that are present. The bound proteins are washed with the provided wash buffer, and are then eluted such that they can be used in downstream applications. The purified proteins can be used in a number of downstream applications including SDS-PAGE analysis or Western blots.

Advantages

- Fast and easy processing using either a vacuum manifold or centrifugation
- All 96-Well Filter Plate for RNA purification and protein purification provided
- Sequentially isolate nucleic acids and proteins from a single lysate – no need to split the lysate
- Isolate total RNA, from large rRNA down to microRNA (miRNA)
- No phenol or chloroform extractions
- Isolate high quality total RNA
- High yields of isolated proteins

Specifications

Kit Specifications	
Binding Capacity Per Well	50 μg for RNA 150 μg for protein
Maximum Loading Volume Per Well	500 μL
Size of RNA Purified	All sizes, including small RNA (<200 nt)
Maximum Amount of Starting Material: Animal Cells Animal Tissues Blood Bacteria Yeast Fungi Plant Tissues	 1 x 10^6 cells 10 mg 100 μL 1 x 10^9 cells 1 x 10^8 cells 40 mg 40 mg
Time to Complete 10 Purifications	25 minutes
Average Yields* HeLa Cells (1 x 10^6 cells) HeLa Cells (1 x 10^6 cells) Liver (5 mg) Liver (5 mg)	 15 μg RNA 150 μg protein 12.5 μg RNA 55 μg protein

***** *average yields will vary depending upon a number of factors including species, growth conditions used and developmental stage.*

Kit Components

Component	Product # 37900 (2 x 96 samples)
Lysis Solution	2 x 40 mL
Wash Solution	2 x 30 mL
Elution Solution	2 x 20 mL
Protein Wash Buffer	2 x 45 mL
Protein pH Binding Buffer	2 x 4 mL
Protein Elution Buffer	2 x 20 mL
Protein Neutralizer	2 x 2 mL
Protein Loading Dye	2 x 2 mL
96-Well Filter Plate	2
Adhesive Tape	4
96-Well Collection Plate	2
96-Well Elution Plate	4
Product Insert	1

Storage Conditions and Product Stability
All solutions should be kept tightly sealed and stored at room temperature. These reagents should remain stable for at least 1 year in their unopened containers. The Protein Loading Dye should be stored at -20°C after the addition of DL-Dithiothreitol (DTT).

Precautions and Disclaimers
This kit is designed for research purposes only. It is not intended for human or diagnostic use.

Ensure that a suitable lab coat, disposable gloves and protective goggles are worn when working with chemicals. For more information, please consult the appropriate Material Safety Data Sheets (MSDSs). These are available as convenient PDF files online at *www.norgenbiotek.com*.

Blood of all human and animal subjects is considered potentially infectious. All necessary precautions recommended by the appropriate authorities in the country of use should be taken when working with whole blood.

The **Lysis Solution** contains guanidine salts, and should be handled with care. Guanidine salt forms highly reactive compounds when combined with bleach, thus care must be taken to properly dispose of any of these solutions.

Technical Support
Contact our Technical Support Team between the hours of 8:30 and 5:30 (Eastern Standard Time) at (905) 227-8848 or Toll Free at 1-866-667-4362.

Technical support can also be obtained from our website (www.norgenbiotek.com) or through email at techsupport@norgenbiotek.com.

Customer-Supplied Reagents and Equipment
You must have the following in order to use the RNA/Protein Purification 96-Well Kit:

For All Protocols
- For ***Vacuum Format***:
  - Vacuum manifold with vacuum pump capable of generating a minimum pressure of -650 mbar or -25 in. Hg (such as Whatman UniVac 3 Vacuum to Collect Manifold)
  - Sealing tape or pads
- For ***Centrifuge Format***:
  - Centrifuge with rotor for 96-well plate assembly (such as Thermo Fisher IEC Centra CL3 series or Beckman GS-15R)
- 95 - 100% ethanol
- Isopropanol
- DL-Dithiothreitol (DTT)
- β-mercaptoethanol (optional)
- Molecular Biology Grade Water
- Deep (1 mL to 2 mL volume) 96-Well Plate or microcentrifuge tubes for flowthrough collection and subsequent pH adjustment for protein purification.

For Animal Cell Protocol
- PBS (RNase-free)

For Animal Tissue Protocol

- Liquid nitrogen
- Mortar and pestle

For Bacterial Protocol

- Lysozyme-containing TE Buffer:
    - For Gram-negative bacteria, 1 mg/mL lysozyme in TE Buffer
    - For Gram-positive bacteria, 3 mg/mL lysozyme in TE Buffer

For Yeast Protocol

- Resuspension Buffer with Lyticase:
    - 50 mM Tris pH 7.5
    - 10 mM EDTA
    - 1 M Sorbital
    - 1 unit/μL Lyticase

For Fungi Protocol

- Liquid nitrogen
- Mortar and pestle

For Plant Protocol

- Liquid nitrogen
- Mortar and pestle

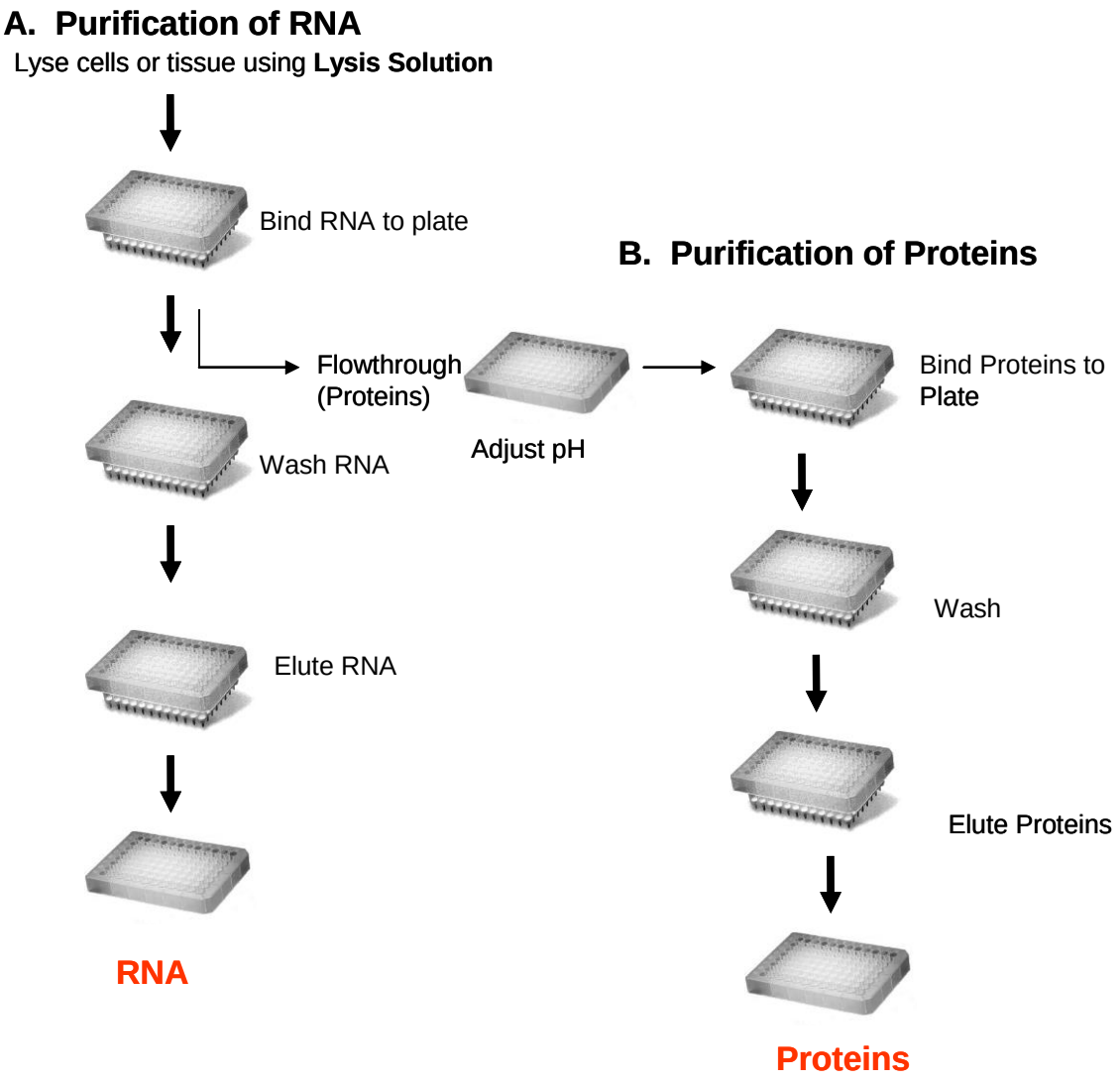
Working with RNA

RNases are very stable and robust enzymes that degrade RNA. Autoclaving solutions and glassware is not always sufficient to actively remove these enzymes. The first step when preparing to work with RNA is to create an RNase-free environment. The following precautions are recommended as your best defense against these enzymes.

- The RNA area should be located away from microbiological work stations
- Clean, disposable gloves should be worn at all times when handling reagents, samples, pipettes, disposable tubes, etc. It is recommended that gloves are changed frequently to avoid contamination
- There should be designated solutions, tips, tubes, lab coats, pipettes, etc. for RNA only
- All RNA solutions should be prepared using at least 0.05% DEPC-treated autoclaved water or molecular biology grade nuclease-free water
- Clean all surfaces with commercially available RNase decontamination solutions
- When working with purified RNA samples, ensure that they remain on ice during downstream applications

Flow Chart

Procedure for Purifying Total RNA and Proteins using
Norgen's RNA/Protein Purification 96-Well Kit



Procedures

For *Vacuum Manifold*: All vacuum steps are performed at room temperature. The correct pressure can be calculated using the conversions:

1 **mbar** = 100 **Pa** = 0.0394 **in. Hg** = 0.75 **mm Hg** = 0.0145 **psi**

For *Centrifugation*: All centrifugation steps are performed at room temperature. The correct rpm can be calculated using the formula:

$$RPM = \sqrt{\frac{RCF}{(1.118 \times 10^{-5})\,(r)}}$$

where *RCF* = required gravitational acceleration (relative centrifugal force in units of g); *r* = radius of the rotor in cm; and *RPM* = the number of revolutions per minute required to achieve the necessary *g*-force.

NOTE: This procedure is written in two steps. Section 1 contains the protocols for preparation of lysate from different types of starting materials. Please ensure that the proper protocol is followed for your sample. The user then carry out total RNA and total protein purification using either Section 2 with vacuum or Section 3 with centrifugation.

Section 1. Preparation of Lysate From Various Cell Types

Notes Prior to Use for all RNA/Protein Purification Procedures

- The steps for preparing the lysate are different depending on the starting material **(Step 1).** However, the subsequent steps are the same in all cases **(Steps 2 – 9)**.
- Please ensure that the correct procedure for preparing the lysate from your starting material is followed.
- Ensure that all solutions are at room temperature prior to use.
- Prepare a working concentration of the **Wash Solution** by adding 90 mL of 95% ethanol (provided by the user) to the supplied bottle containing the concentrated **Wash Solution**. This will give a final volume of 120 mL. The label on the bottle has a box that may be checked to indicate that the ethanol has been added.
- The volumes stated in each procedure for lysate preparation are the volumes required to prepare samples for each well of the 96-well plate.
- **Optional:** The use of β-mercaptoethanol in lysis is highly recommended for most animal tissues, particularly those known to have a high RNAse content (ex: pancreas), as well as for most plant tissues. It is also recommended for users who wish to isolate RNA for sensitive downstream applications. Add 10 µL of β-mercaptoethanol (provided by the user) to each 1 mL of Lysis Solution required. β-mercaptoethanol is toxic and should be dispensed in a fume hood. Alternatively, the lysis solution can be used as provided.
- It is important to work quickly during this procedure.

Section 1A. Preparation of Lysate From Various Cell Types

1A. Lysate Preparation from Cultured Animal Cells

Notes Prior to Use

- The recommended input is 5 x 10^5 cells per well of the 96-Well Filter Plate. However, up to 1 x 10^6 cells may be processed for most cell lines.
- A hemocytometer can be used in conjunction with a microscope to count the number of cells. As a general guideline, a confluent 3.5 cm plate of HeLa cells will contain 10^6 cells.

- Cell pellets can be stored at -70°C for later use or used directly in the procedure. Determine the number of cells present before freezing.
- Frozen pellets should be stored for no longer than 2 weeks to ensure that the integrity of the RNA is not compromised.
- Frozen cell pellets should not be thawed prior to beginning the protocol. Add the Lysis Solution directly to the frozen cell pellet **(Step 1A(ii) d).**

1A (i). Cell Lysate Preparation from Cells Growing in a Monolayer

a. Aspirate media and wash cell monolayer with an appropriate amount of PBS. Aspirate PBS.
b. Add 250 µL of **Lysis Solution** directly to culture plate.
c. Lyse cells by gently tapping culture dish and swirling buffer around plate surface for five minutes.
d. Transfer lysate to a microcentrifuge tube.

   Note: At this stage the lysate may be stored at -70°C, such that the RNA purification can be performed at a later time.

e. Add 100 µL of isopropanol (provided by the user) to the lysate. Mix by vortexing for 10 seconds. **Proceed to Step 2.**

1A (ii). Cell Lysate Preparation from Cells Growing in Suspension and Lifted Cells

a. Transfer cell suspension to an RNase-free tube (not provided) and centrifuge in a microcentrifuge at no more than 200 *x g* (~2,000 RPM) for 10 minutes to pellet cells.
b. Carefully decant the supernatant to ensure that the pellet is not dislodged. Wash the cell pellet with an appropriate amount of PBS. Centrifuge at 200 x g (~2,000 RPM) for another 5 minutes.
c. Carefully decant the supernatant. A few µL of PBS may be left behind with the pellet in order to ensure that the pellet is not dislodged.
d. Add 250 µL of **Lysis Solution** to the pellet. Lyse cells by vortexing for 15 seconds. Ensure that the entire pellet is completely dissolved before proceeding to the next step.

   Note: At this stage the lysate may be stored at -70°C, such that the RNA purification can be performed at a later time.

e. Add 100 µL of isopropanol (provided by the user) to the lysate. Mix by vortexing for 10 seconds. **Proceed to Step 2.**

1B. Lysate Preparation from Animal Tissues

Notes Prior to Use

- RNA in animal tissues is not protected after harvesting until it is disrupted and homogenized. Thus it is important that the procedure is carried out as quickly as possible, particularly the Cell Lysate Preparation step.
- Fresh or frozen tissues may be used for the procedure. Tissues should be flash-frozen in liquid nitrogen and transferred immediately to a -70°C freezer for long-term storage. Tissues may be stored at -70°C for several months. When isolating total RNA from frozen tissues ensure that the tissue does not thaw during weighing or prior to homogenization.
- The optimal amount of non-fibrous tissue be used per well of the 96-Well Filter Plate is up to 8 mg. However, for most tissues (except tissues with high cell number such as liver and spleen), up to 10 mg could be processed. For fibrous tissue such as heart, a maximum of 2 mg is recommended

1B. Cell Lysate Preparation from Animal Tissues

a. Excise the tissue sample from the animal.
b. Determine the amount of tissue by weighing. It is recommended that no more than 10 mg of tissue be used for each well of the 96-Well Filter Plate.
c. Transfer the tissue samples to appropriate vessels for the desired disruption method.
d. Add 300 µL of **Lysis Solution** to each tissue sample.

Note: Ensure that frozen tissues do not thaw during weighing or prior to the addition of Lysis Solution. For maximum RNA recovery, homogenize frozen tissues to fine powder in liquid nitrogen prior to the addition of **Lysis Solution.**

e. Homogenize the tissues using the appropriate cell disruption tool.

Note: Thorough homogenization is required for optimal performance. For tissue inputs of ≤ 8 mg, it is not required to perform centrifugation to remove cell debris if the homogenization is complete. For tissue inputs larger than 8 mg, or if incomplete cell disruption is suspected, centrifuge the lysate at maximum speed for 2 minutes in an appropriate centrifuge. Transfer the supernatant to a new 96-well microplate.

Note: At this stage the lysate may be stored at -70°C, such that the RNA purification can be performed at a later time.

f. Add 120 µL of isopropanol (provided by the user) to each tissue sample. Mix by pipetting up and down a few times.

1C. Lysate Preparation from Blood

Notes Prior to Use

- Blood of all human and animal subjects is considered potentially infectious. All necessary precautions recommended by the appropriate authorities in the country of use should be taken when working with whole blood.
- It is recommended that no more than 100 µL of blood be used in order to prevent clogging of the column.
- We recommend the use of this kit to isolate RNA from non-coagulating fresh blood using EDTA as the anti-coagulant.

1C. Cell Lysate Preparation from Blood

a. Transfer up to 100 µL of non-coagulating blood to an RNase-free microcentrifuge tube (not provided).
b. Add 200 µL of **Lysis Solution** to the blood. Lyse cells by vortexing for 15 seconds. Ensure that mixture becomes transparent before proceeding to the next step.

Note: At this stage the lysate may be stored at -70°C, such that the RNA purification can be performed at a later time.

c. Add 120 µL of isopropanol (provided by the user) to the lysate. Mix by vortexing for 10 seconds. **Proceed to Step 2.**

1D. Lysate Preparation from Bacteria

Notes Prior to Use

- Prepare the appropriate lysozyme-containing TE Buffer as indicated in Table 1. This solution should be prepared with sterile, RNAse-free TE Buffer, and kept on ice until needed. These reagents are to be provided by the user.

- It is recommended that no more than 10^9 bacterial cells be used in this procedure. Bacterial growth can be measured using a spectrophotometer. As a general rule, an *E. coli* culture containing 1 x 10^9 cells/mL has an OD_{600} of 1.0.
- For RNA isolation, bacteria should be harvested in log-phase growth.
- Bacterial pellets can be stored at -70°C for later use, or used directly in this procedure.
- Frozen bacterial pellets should not be thawed prior to beginning the protocol. Add the Lysozyme-containing TE Buffer directly to the frozen bacterial pellet **(Step 1Dc)**.

1D. Cell Lysate Preparation from Bacteria

a. Pellet bacteria by centrifugation of a 96-well block at 3,000 *x g* (3,000 RPM) for 5 mintues or centrifugation of microcentrifuge tubes at 14,000 *x g* (~14,000 RPM) for 1 minute.
b. Decant supernatant, and carefully remove any remaining media by aspiration.
c. Resuspend the bacteria thoroughly in 100 µL of the appropriate lysozyme-containing TE Buffer (see Table 1) by vortexing. Incubate at room temperature for the time indicated in Table 1.
d. Add 200 µL of Lysis Solution and vortex vigorously for at least 10 seconds.

   Note: At this stage the lysate may be stored at -70°C, such that the RNA purification can be performed at a later time.

e. Add 120 µL of isopropanol (provided by the user) to the lysate. Mix by vortexing for 10 seconds. **Proceed to Step 2.**

Table 1: Incubation Time for Different Bacterial Strains

Bacteria Type	Lysozyme Concentration in TE Bufffer	Incubation Time
Gram-negative	1 mg/mL	5 min
Gram-positive	3 mg/mL	10 min

1E. Lysate Preparation from Yeast

Notes Prior to Use

- Prepare the appropriate amount of Lyticase-containing Resuspension Buffer, considering that 500 µL of buffer is required for each preparation. The Resuspension Buffer should have the following composition: 50 mM Tris, pH 7.5, 10 mM EDTA, 1M Sorbital, 0.1% β-mercaptoethanol and 1 unit/µL Lyticase. This solution should be prepared with sterile, RNAse-free reagents, and kept on ice until needed. These reagents are to be provided by the user.
- It is recommended that no more than 10^7 yeast cells or 1 mL of culture be used for this procedure.
- For RNA isolation, yeast should be harvested in log-phase growth.
- Yeast can be stored at -70°C for later use, or used directly in this procedure.
- Frozen yeast pellets should not be thawed prior to beginning the protocol. Add the Lyticase-containing Resuspension Buffer directly to the frozen yeast pellet **(Step 1Ec)**.

1E. Cell Lysate Preparation from Yeast

a. Pellet yeast by centrifugation of a 96-well block at 3,000 *x g* (3,000 RPM) for 5 mintues or centrifugation of microcentrifuge tubes at 14,000 *x g* (~14,000 RPM) for 1 minute.
b. Decant supernatant, and carefully remove any remaining media by aspiration.
c. Resuspend the yeast thoroughly in 500 µL of Lyticase-containing Resuspension Buffer by vortexing. Incubate at 37°C for 10 minutes.

d. Pellet the spheroplasts. Centrifuge a 96-well block at 650 x g (~800 - 1,000 RPM) for 3 minutes or microcentrifuge tubes at 200 *x g* (~2,000 RPM) for 3 minutes and remove the supernatant by pipetting.
e. Add 250 µL of Lysis Solution and vortex vigorously for at least 10 seconds.

   Note: At this stage the lysate may be stored at -70°C, such that the RNA purification can be performed at a later time.

f. Add 100 µL of isopropanol (provided by the user) to the lysate. Mix by vortexing for 10 seconds. **Proceed to Step 2.**

1F. Lysate Preparation from Fungi

Notes Prior to Use

- Fresh or frozen fungi may be used for this procedure. Fungal tissue should be flash-frozen in liquid nitrogen and transferred immediately to a -70°C freezer for long-term storage. Fungi may be stored at -70°C for several months. Do not allow frozen tissues to thaw prior to grinding with the mortar and pestle in order to ensure that the integrity of the RNA is not compromised.
- It is recommended that no more than 40 mg of fungi be used for this procedure in order to prevent clogging of the column.

1F. Cell Lysate Preparation from Fungi

a. Determine the amount of fungi by weighing. It is recommended that no more than 40 mg of fungi be used for the protocol.
b. Transfer the fungus into a mortar that contains an appropriate amount of liquid nitrogen to cover the sample. Grind the fungus thoroughly using a pestle.
c. Allow the liquid nitrogen to evaporate, without allowing the tissue to thaw.
d. Add 300 µL of **Lysis Solution** to the tissue sample and continue to grind until the sample has been homogenized.
e. Using a pipette, transfer the lysate into an RNase-free microcentrifuge tube (not provided).
f. Spin the lysate for 2 minutes to pellet any cell debris. Transfer the supernatant to another RNase-free microcentrifuge tube.

   Note: At this stage the lysate may be stored at -70°C, such that the RNA purification can be performed at a later time.

g. Add 100 µL of isopropanol (provided by the user) to the lysate. Mix by vortexing for 10 seconds. **Proceed to Step 2.**

1G. Lysate Preparation from Plant

Notes Prior to Use

- The maximum recommended input of plant tissue is 40 mg or 4 x 10^6 plant cells.
- Both fresh and frozen plant samples can be used for this protocol. Samples should be flash-frozen in liquid nitrogen and transferred immediately to a -70°C freezer for long-term storage. Do not allow frozen tissues to thaw prior to grinding with the mortar and pestle in order to ensure that the integrity of the RNA is not compromised.

1G. Cell Lysate Preparation from Plant

a. Transfer ≤40 mg of plant tissue or 4 x 10^6 plant cells into a mortar that contains an appropriate amount of liquid nitrogen to cover the sample. Grind the sample into a fine powder using a pestle in liquid nitrogen.

   Note: If stored frozen samples are used, do not allow the samples to thaw before transferring to the liquid nitrogen.

b. Allow the liquid nitrogen to evaporate, without allowing the tissue to thaw.
c. Add 300 µL of **Lysis Solution** to the tissue sample and continue to grind until the sample has been homogenized.
d. Using a pipette, transfer the lysate into an RNase-free microcentrifuge tube (not provided).
e. Spin the lysate for 2 minutes to pellet any cell debris. Transfer the supernatant to another RNase-free microcentrifuge tube.

   Note: At this stage the lysate may be stored at -70°C, such that the RNA purification can be performed at a later time.

f. Add 100 µL of isopropanol (provided by the user) to the lysate. Mix by vortexing for 10 seconds. **Proceed to Step 2.**

Note: The purification of total RNA from the lysate prepared in Section 1 could be performed using either a vacuum manifold or centrifugation. For purification using vacuum, please follow the procedure outlined in Section 2A. For purification using centrifugation, please follow the procedure outlined in Section 2B

Section 2: RNA/Protein Purification from All Types of Lysate using Vacuum

Note: The remaining steps of the procedure for the purification of total RNA/proteins using a vacuum manifold are the same from this point forward for all the different types of lysate.

A. Total RNA Purification Using Vacuum Manifold

2. Binding RNA to 96-Well Filter Plate

a. Assemble the 96-Well Filter Plate and the vacuum manifold according to manufacturer's recommendations.

   Note: The provided 96-Well Collection Plate can be used as the collection/waste tray if desired.

b. Apply the lysate with the isopropanol (from **Step 1**) into each well of the 96-Well Filter Plate. Tape the plate or any unused wells using sealing tape or pads (provided by the user) according to the vacuum manifold manufacturer's recommendations. Apply vacuum for 2 minutes.

   Note: Depending on the starting material, a small quantity of precipitates may appear in the lysate-ethanol mix. No additional step is required to remove these precipitates prior to application of the mixture to the wells.

c. Turn off vacuum and ventilate the manifold. **Retain the flowthrough for Protein Purification (Section 2B) by transferring to another 96-well plate (not provided) or**

microcentrifuge tubes (not provided). The flowthough contains the proteins and should be stored on ice or at -20°C until the Protein Purification protocol is carried out. Reassemble the 96-Well Filter Plate and the vacuum manifold.

Note: Ensure that all of the lysate from each well has passed through into the collection/waste tray. If the entire lysate volume has not passed, apply vacuum for an additional 2 minutes.

Optional Step:

Norgen's RNA/Protein Purification 96-Well Kit isolates total RNA with minimal amounts of genomic DNA contamination. However, an optional **On-Plate DNA Removal Protocol** is provided in Appendix A for maximum removal of residual DNA that may affect sensitive downstream applications. This step should be performed at this point in the protocol.

3. RNA Wash

a. Apply 400 µL of **Wash Solution** to each well of the 96-Well Filter Plate. Tape the plate or any unused wells using sealing tape or pads (provided by the user) according to the vacuum manifold manufacturer's recommendations. Apply vacuum for 2 minutes.

Note: Ensure the entire wash solution has passed through into the collection/waste tray by inspecting the 96-Well Filter Plate. If the entire wash volume has not passed, apply vacuum for an additional 2 minutes.

b. Turn off vacuum and ventilate the manifold. Discard the flowthrough.
c. Reassemble the 96-Well Filter Plate and the vacuum manifold. Repeat steps **3a** and **3b** to wash column for a second time.
d. Reassemble the 96-Well Filter Plate and the vacuum manifold. Repeat steps **3a** and **3b** to wash column for a third time.
e. Pat the bottom of the 96-Well Filter Plate dry. Reassemble the 96-Well Filter Plate and the vacuum manifold. Apply vacuum for an additional 5 minutes in order to completely dry the plate.
f. Turn off vacuum and ventilate the manifold. **Retain the 96-Well Collection Plate (if used) for Protein Purification.**

4. RNA Elution

a. Replace the collection/waste tray in the vacuum manifold with the provided 96-Well Elution Plate. Complete the vacuum manifold assembly with the 96-Well Filter Plate.
b. Add 75 µL of **Elution Solution** to each well of the plate.
c. Apply vacuum for 2 minutes.

Note: Ensure the entire **Elution Solution** has passed through into the elution plate by inspecting the 96-Well Filter Plate. If the entire elution volume has not passed, apply vacuum for an additional 2 minutes.

d. **Retain the 96-Well Filter Plate for Protein Purification.**

5. Storage of RNA

Use the provided adhesive tape to seal the 96-Well Elution Plate. The purified RNA samples may be stored at –20°C for a few days. It is recommended that samples be placed at –70°C for long term storage.

B. Total Protein Purification from All Cell Types Using Vacuum

Notes Prior to Use

- At this point, the proteins that are present in the flowthrough from the RNA Binding Step **(Step 2 above)** can be loaded directly onto an SDS-PAGE gel for visual analysis, or the proteins can be further purified using the protocol below.
- Add 93 mg of DL-Dithiothreitol (DTT, not provided) to the **Protein Loading Dye**. The Protein Loading Dye should be stored at -20°C after the addition of DTT. The label on the bottle has a box that may be checked to indicate that DTT has been added.
- For direct running on a gel, the provided Protein Loading Dye should be used instead of regular SDS-PAGE Loading Buffer in order to prevent any precipitates from forming. Add 1 volume of the Protein Loading Dye to the sample and boil for 2 minutes before loading.

6. pH Adjustment of Lysate

a. Use 100 µL of flowthrough from the RNA Binding Step **(Step 2c above)**.

Note: Up to 200 µL of flowthrough may be used. However, the recovery efficiency may be decreased when processing a larger volume.

b. Adjust volume to 400 µL with Molecular Biology Grade Water.

c. Add 16 µL of **pH Binding Buffer**. Mix contents well.

Note: If the entire lysate is to be purified, repeat step **6a** to **6c** with the remaining lysate.

7. Protein Binding

a. Apply the pH-adjusted protein samples into each well of the 96-Well Filter Plate. Tape the plate or any unused wells using sealing tape or pads (provided by the user) according to the vacuum manifold manufacturer's recommendations. Apply vacuum for 2 minutes.

b. Turn off vacuum and ventilate the manifold. Discard the flowthrough.

c. Depending on your sample volume, repeat steps **7a** and **7b** until the entire protein sample has been loaded onto the column.

8. Column Wash

a. Apply 400 µL of **Protein Wash Buffer** to to each well of the 96-Well Filter Plate. Tape the plate or any unused wells using sealing tape or pads (provided by the user) according to the vacuum manifold manufacturer's recommendations. Apply vacuum for 2 minutes.

b. Turn off vacuum and ventilate the manifold. Discard the flowthrough. Reassemble the 96-Well Filter Plate and the vacuum manifold.

c. Turn off vacuum and ventilate the manifold. Pat the bottom of the 96-Well Filter Plate dry. Reassemble the 96-Well Filter Plate and the vacuum manifold. Apply vacuum for an additional 5 minutes in order to completely dry the plate.

d. Turn off vacuum and ventilate the manifold.

9. Protein Elution and pH Adjustment

The supplied **Protein Elution Buffer** consists of 10 mM sodium phosphate pH 12.5.

a. Add 9.3 µL of **Neutralizer** to the appropriate wells of a new 96-Well Elution Plate.

b. Stack the 96-Well Filter Plate on top of the 96-Well Elution Plate containing the **Neutralizer** to complete the vacuum manifold set up.

c. Apply 100 µL of the **Protein Elution Buffer** to the wells of the 96-Well Filter Plate

d. Apply vacuum for 2 minutes to elute the proteins.

Note: Ensure the entire **Protein Elution Buffer** has passed through into the elution plate by inspecting the 96-Well Filter Plate. If the entire elution volume has not passed, apply vacuum for an additional 2 minutes

e. Gently agitate the elution plate to mix the eluent with the **Neutralizer.**

Section 3: RNA/Protein Purification from All Types of Lysate using Centrifugation

Note: The remaining steps of the procedure for the purification of total RNA and proteins using centrifugation are the same from this point forward for all the different types of lysate.

A. RNA Purification Using Centrifugation

2. Binding RNA to 96-Well Filter Plate

a. Place the 96-Well Filter Plate on top of a provided 96-Well Collection Plate.

b. Apply the lysate with the isopropanol (from **Step 1**) into each well of the 96-Well Filter Plate. Centrifuge the assembly at maximum speed or 3,000 *x g* (~3,000 RPM) for 2 minutes.

Note: Depending on the starting material, a small quantity of precipitates may appear in the lysate-ethanol mix. No additional step is required to remove these precipitates prior to application to the wells

c. **Retain the flowthrough for Protein Purification (Section 3B) by transferring to another 96-well plate (not provided) or microcentrifuge tubes (not provided). The flowthough contains the proteins and should be stored on ice or at -20°C until the Protein Purification protocol is carried out.** Reassemble the the 96-Well Filter Plate and the bottom plate.

Note: Ensure that all of the lysate from each well has passed through into the bottom plate. If the entire lysate volume has not passed, centrifuge for an additional 2 minutes.

Optional Step:

Norgen's RNA/Protein Purification 96-Well Kit isolates total RNA with minimal amounts of genomic DNA contamination. However, an optional **On-Plate DNA Removal Protocol** is provided in Appendix A for maximum removal of residual DNA that may affect sensitive downstream applications. This step should be performed at this point in the protocol

3. RNA Wash

a. Apply 400 µL of **Wash Solution** to each well of the 96-Well Filter Plate. Centrifuge the assembly at maximum speed or 3,000 *x g* (~3,000 RPM) for 2 minutes.

Note: Ensure the entire wash solution has passed through into the bottom plate by inspecting the 96-Well Filter Plate. If the entire wash volume has not passed, centrifuge for an additional 2 minutes.

b. Discard the flowthrough. Reassemble the 96-Well Filter Plate and the bottom plate.

c. Repeat steps **3a** and **3b** to wash column for a second time.

d. Repeat steps **3a** and **3b** to wash column for a third time.

e. Pat the bottom of the 96-Well Filter Plate dry. Reassemble the 96-Well Filter Plate and the bottom plate. Centrifuge the assembly at maximum speed or 3,000 *x g* (~3,000

RPM) for 5 minutes in order to completely dry the plate. **Retain the 96-Well Collection Plate for Protein Purification.**

4. RNA Elution

a. Stack the 96-Well Filter Plate on top of the 96-Well Elution Plate.
b. Add 75 µL of **Elution Solution** to each well of the 96-Well Filter Plate.
c. Centrifuge the assembly at maximum speed or 3,000 *x g* (~3,000 RPM) for 2 minutes.

Note: Ensure the entire **Elution Solution** has passed through into the elution plate by inspecting the 96-Well Filter Plate. If the entire elution volume has not passed, apply centrifugation for an additional 2 minutes

d. **Retain the 96-Well Filter Plate for Protein Purification.**

5. Storage of RNA

Use the provided adhesive tape to seal the 96-Well Elution Plate. The purified RNA sample may be stored at –20°C for a few days. It is recommended that samples be placed at –70°C for long term storage.

B. Total Protein Purification from All Cell Types Using Centrifugation

Notes Prior to Use

- At this point, the proteins that are present in the flowthrough from the RNA Binding Step **(Step 2 above)** can be loaded directly onto an SDS-PAGE gel for visual analysis, or the proteins can be further purified using the protocol below.
- Add 93 mg of DL-Dithiothreitol (DTT, not provided) to the **Protein Loading Dye**. The Protein Loading Dye should be stored at -20°C after the addition of DTT. The label on the bottle has a box that may be checked to indicate that DTT has been added
- For direct running on a gel, the provided Protein Loading Dye should be used instead of regular SDS-PAGE Loading Buffer in order to prevent any precipitates from forming. Add 1 volume of the Protein Loading Dye to the sample and boil for 2 minutes before loading.

6. pH Adjustment of Lysate

a. Use 100 µL of flowthrough from the RNA Binding Step **(Step 2c above)**.

Note: Up to 200 µL of flowthrough may be used. However, the recovery efficiency may be decreased when processing a larger volume.

b. Adjust volume to 400 µL with Molecular Biology Grade Water.
c. Add 16 µL of **pH Binding Buffer**. Mix contents well.

Note: If the entire lysate is to be purified, repeat step **6a** to **6c** with the remaining lysate.

7. Protein Binding

a. Apply the pH-adjusted protein samples into each well of the 96-Well Filter Plate. Centrifuge the assembly at maximum speed or 3,000 *x g* (~3,000 RPM) for 2 minutes.
b. Discard the flowthrough. Reassemble the 96-Well Filter Plate with the collection plate.
c. Depending on your sample volume, repeat steps **7a** and **7b** until the entire protein sample has been loaded onto the column.

8. Column Wash

a. Apply 400 µL of **Protein Wash Buffer** to to each well of the 96-Well Filter Plate. Centrifuge the assembly at maximum speed or 3,000 x g (~3,000 RPM) for 2 minutes.
b. Discard the flowthrough and reassemble the spin column with its collection tube.

c. Inspect the column to ensure that the liquid has passed through into the collection tube. There should be no liquid in the column. If necessary, spin for an additional minute to dry.

9. Protein Elution and pH Adjustment

The supplied **Protein Elution Buffer** consists of 10 mM sodium phosphate pH 12.5.

a. Add 9.3 µL of **Neutralizer** to the appropriate wells of a new 96-Well Elution Plate.
b. Stack the 96-Well Filter Plate on top of the 96-Well Elution Plate containing the **Neutralizer**.
c. Apply 100 µL of the **Protein Elution Buffer** to the wells of the 96-Well Filter Plate
d. Centrifuge the assembly at maximum speed or 3,000 *x g* (~3,000 RPM) for 2 minutes to elute bound proteins.

Note: Ensure the entire **Protein Elution Buffer** has passed through into the elution plate by inspecting the 96-Well Filter Plate. If the entire elution volume has not passed, apply centrifugation for an additional 2 minutes

e. Gently agitate the elution plate to mix the eluent with the **Neutralizer.**

Appendix A

Protocol for Optional On-Plate DNA Removal

Norgen's RNA/Protein Purification 96-Well Kit isolates total RNA with minimal amounts of genomic DNA contamination. However, an optional protocol is provided below for maximum removal of residual DNA that may affect sensitive downstream applications. It is recommended that Norgen's RNase-Free DNase I Kit (Product # 25710) be used for this step.

1. For every on-column reaction to be performed, prepare a mix of 10 µL of **DNase I** and 65 µL of **Enzyme Incubation Buffer** using Norgen's RNase-Free DNase I Kit (Product # 25710). Mix gently by inverting the tube a few times. **DO NOT VORTEX**.

   Note: If using an alternative DNase I, prepare a working stock of 0.25 Kunitz unit/µL RNase-free DNase I solution according to the manufacturer's instructions. A 75 µL aliquot is required for each column to be treated.

2. Perform the appropriate Total RNA Purification Procedure for your starting material up to and including "**Binding RNA to 96-Well Filter Plate**" (Steps 1 and 2 of all protocols)
3. ***For Vacuum Manifold*****:** Apply 400 µL of **Wash Solution** to each well of the 96-Well Filter Plate. Tape the plate or any unused wells using sealing tape or a pad (provided by the user) according to the vacuum manifold manufacturer's recommendations. Apply vacuum for 2 minutes.
   For Centrifugation**:** Apply 400 µL of **Wash Solution** to each well of the 96-Well Filter Plate. Centrifuge the assembly at maximum speed or 3,000 *x g* (~3,000 RPM) for 2 minutes.
4. Discard the flowthrough. Reassemble the 96-Well Filter Plate with the vacuum manifold or the bottom plate.
5. Apply 75 µL of the RNase-free DNase I solution prepared in Step 1 to each well of the 96-Well Filter Plate.
   For Vacuum Manifold**:** Apply vacuum for 30 seconds.
   For Centrifugation**:** Centrifuge the assembly at maximum speed or 3,000 *x g* (~3,000 RPM) for 30 seconds.

6. After the centrifugation or vacuum in Step 5, pipette the flowthrough that is present in the collection plate back onto the top of the column.

   Note: Ensure Step 6 is performed in order to ensure maximum DNase activity and to obtain maximum yields of RNA, in particular for small RNA species

7. Incubate the assembly at 25 - 30°C for 15 minutes.
8. Without any further centrifugation, proceed directly to "**RNA Wash**" Section 2A, Step 3c for **Vacuum Manifold** procedure or Section 2B, Step 3c for **Centrifugation** procedure.

Troubleshooting Guide

Problem	Possible Cause	Solution and Explanation
Poor RNA Recovery	Incomplete lysis of cells or tissue	Ensure that the appropriate amount of Lysis Solution was used for the amount of cells or tissue.
	Well has become clogged	Do not exceed the recommended amounts of starting materials. The amount of starting material may need to be decreased if the column shows clogging below the recommended levels. See also "Clogged Well" below.
	An alternative elution solution was used	It is recommended that the Elution Solution supplied with this kit be used for maximum RNA recovery.
	Alcohol was not added to the lysate	Ensure that the appropriate amount of isopropanol is added to the lysate before binding to the column.
	Ethanol was not added to the Wash Solution	Ensure that 90 mL of 95% ethanol is added to the supplied Wash Solution prior to use.
	Low RNA content in cells or tissues used	Different tissues and cells have different RNA contents, and thus the expected yield of RNA will vary greatly from these different sources. Please check literature to determine the expected RNA content of your starting material.
	Cell Culture: Cell monolayer was not washed with PBS	Ensure that the cell monolayer is washed with the appropriate amount of PBS in order to remove residual media from cells.
	Yeast: Lyticase was not added to the Resuspension Buffer	Ensure that the appropriate amount of lyticase is added when making the Resuspension Buffer.
	Bacteria and Yeast: All traces of media not removed	Ensure that all media is removed prior to the addition of the lysis solution through aspiration.

Problem	Possible Cause	Solution and Explanation
Clogged Well	Insufficient solubilization of cells or tissues	Ensure that the appropriate amount of lysis buffer was used for the amount of cells or tissue.
	Insufficient Vacuum	Ensure that a vacuum pressure of at least -650 mbar or -25 in. Hg is developed
	Maximum number of cells or amount of tissue exceeds kit specifications	Refer to specifications to determine if amount of starting material falls within kit specifications
Clogged Well	High amounts of genomic DNA present in sample	The lysate may be passed through a 25 gauge needle attached to a syringe 5-10 times in order to shear the genomic DNA prior to loading onto the column.
	Centrifuge temperature too low	Ensure that the centrifuge remains at room temperature throughout the procedure. Temperatures below 20°C may cause precipitates to form that can cause the columns to clog.
RNA is Degraded	RNase contamination	RNases may be introduced during the use of the kit. Ensure proper procedures are followed when working with RNA. Please refer to "*Working with RNA"* at the beginning of this user guide.
	Procedure not performed quickly enough	In order to maintain the integrity of the RNA, it is important that the procedure be performed quickly. This is especially important for the Cell Lysate Preparation Step in the Animal Tissue protocol, since the RNA in animal tissues is not protected after harvesting until it is disrupted and homogenized.
	Improper storage of the purified RNA	For short term storage RNA samples may be stored at –20°C for a few days. It is recommended that samples be stored at –70°C for longer term storage.
	DNase I used may not be RNase-free	Ensure that the optional DNase I being used with this kit is RNase-free in order to prevent possible problems with RNA degradation. Norgen's RNase-Free DNase I Kit (Cat# 25710) is recommended for this step.
	Lysozyme or lyticase used may not be RNAse-free	Ensure that the lysozyme and lyticase being used with this kit is RNase-free, in order to prevent possible problems with RNA degradation.
RNA is Degraded	Starting material may have a high RNase content	For starting materials with high RNAase content, it is recommended that β-mercaptoethanol be added to the Lysis Solution.
	Frozen tissues or cell pellets were allowed to thaw prior to RNA isolation	Do not allow frozen tissues to thaw prior to grinding with the mortar and pestle in order to ensure that the integrity of the RNA is not compromised.

Problem	Possible Cause	Solution and Explanation
RNA does not perform well in downstream applications	RNA was not washed three times with the provided Wash Solution	Traces of salt from the binding step may remain in the sample if the well is not washed three times with Wash Solution. Salt may interfere with downstream applications, and thus must be washed from the well.
	Ethanol carryover	Ensure that the dry spin under the RNA Wash procedure is performed, in order to remove traces of ethanol prior to elution. Ethanol is known to interfere with many downstream applications.
Poor protein recovery	Incorrect pH adjustment of sample.	Ensure that the pH of the starting protein sample is adjusted to pH 3.5 or lower after the pH Binding Buffer has been added and prior to binding to the column. If necessary, add additional pH Binding Buffer.
	Low protein content in the starting materials	Run a 20 µL fraction from the flowthrough (after RNA binding) on a SDS-PAGE gel to estimate the amount of protein present in the sample. In addition, use the entire flowthrough in protein purification procedure
Proteins are degraded	Eluted protein solution was not neutralized.	Add 9.3 µL of Neutralizer to each 100 µL of eluted protein in order to adjust the pH to neutral. Some proteins are sensitive to high pH, such as the elution buffer at pH 12.5
	Eluted protein was not neutralized quickly enough.	If eluted proteins are not used immediately, degradation will occur. We strongly suggest adding Neutralizer in order to lower the pH.

Related Products	Product #
RNA/DNA/Protein Purification Kit	23500
RNA/Protein Purification Kit	23000
Total RNA Purification Kit	17200
Total RNA Purification 96-Well Kit	24300
RNase-Free DNase I Kit	25710
1kb RNA Ladder	15003
UltraRanger 1kb DNA Ladder	12100

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